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I have developed a series of reagents and a luciferase-based functional assay for identifying and testing ligands for Notch4 receptor. I am in the process of testing several candidate genes for their ability to activate Notch4 receptor.

In addition, I identified a novel negative regulator of Notch activity, SEL-10. Through biochemical studies, I have shown that SEL-10 interacts with Notch in a domain specific and probably phosphorylation-dependent manner. I also provided some evidence indicating that SEL-10 can regulate Notch ubiquitination in the cell. These results support my model that SEL-10 inhibits Notch activity by targeting it for ubiquitin-mediated protein degradation.

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Introduction

I. Nature of the Problem

Notch/lin-12 receptors have been shown in many cases to regulate cell fate decisions. Abnormal activation of some of these receptors is also linked to tumorigenesis. Among all the Notch/lin-12 family members, mouse Notch4/int-3 is the only one that has been reported to be expressed in mammary epithelium, and to regulate mammary gland development and tumorigenesis.

A few ligands of the Notch/lin-12 receptors have been identified and as expected, have been shown to regulate cell fate decisions. However, none of these ligands have been investigated for their role in tumorigenesis or mammary gland development. As for Notch4, the only Notch/lin-12 receptor associated with mammary gland development and tumorigenesis, no ligand has been identified yet.

My proposed study intend to identify Notch4 ligands, study the nature of their interactions with Notch4 receptor and determine their roles in mammary gland development and tumorigenesis. The results of my research will shed light on Notch signaling and mammary gland biology from a new angle.

In addition to the studies on Notch receptors ligands, my collaborators and I identified a novel gene, *sel-10*, as a negative regulator of *Notch/lin-12* activity. Due to the importance of *int-3* as an oncogene involved in mammary tumorigenesis and the potential of *sel-10* as a therapeutic tool against *int-3*, I carried out experiments to understand the

molecular mechanisms by which SEL-10 regulates Notch. My results strongly indicate that SEL-10 inhibits Notch by targeting Notch receptor for ubiquitin-mediated protein degradation. This part of my research, although not originally proposed in my Fellowship Application, has provided the same kind of scientific training required by the Fellowship. The results of this research are presented in this annual report under Specific Aims 3, 4, and 5, which are new additions to the two original Specific Aims.

II. Background

Notch4/int-3 proto-oncogene was cloned by our lab. *Notch4* gene encodes a 220KD protein product, which bears all the hallmarks of a *Notch/lin-12* gene family member. This protein product is expressed in mouse embryos and a number of adult tissues including lung, kidney, heart and mammary gland(1, 2). *In situ* hybridization data showed that Notch4 expression in day 13.5 embryo and adult lung is endothelial cell specific(1). Our unpublished immunofluorescent staining data have confirmed that Notch4 is a cell surface localized protein.

Truncated Notch proteins have been shown to be involved in tumorigenesis. In *Drosophila* and other organisms, truncated Notch proteins containing the intracellular domain behave like constitutively activated receptors(3). The truncated form of *TAN-1* gene was first isolated from patients with acute T lymphoblastic leukemia(4), and was later shown to promote T cell neoplasm in bone marrow reconstitution assay(5) and to transform rat kidney cells in soft-agar assay(6). The truncated *Notch4* gene, *int-3* was identified as a target of integration by mouse mammary tumor virus (MMTV) in mouse mammary tumors. Viral integration into the *int-3* gene results in the expression of a truncated 2.4kb

transcript(7). Transgenic mice that express *int-3* under the control of either MMTV LTR or whey acidic protein (WAP) promoter develop poorly differentiated mammary adenocarcinomas at 100% penetrance(8, 9). Histological examination showed that the mammary glands of the transgenic mice were arrested during development and were lactation deficient(8). int-3 has also been demonstrated to be oncogenic in cultured mammary epithelial cells(7).

Although Notch receptors activated by truncation have been extensively studied for their role in development and tumorigenesis, Notch receptors activated by ligand have never been analyzed in the same scenario. Genetic and molecular analyses have identified several Notch ligands in *Drosophila* and *C. elegans*. *Drosophila Delta* and *Serrate* genes and *C. elegans Lag-2* and *Apx-1* genes encode a family of structurally related ligands for the *Drosophila* Notch and the *C. elegans* lin-12 and glp-1 receptors(10, 11). These ligands are all membrane-bound proteins of which the extracellular domains contain a variable number of EGF-like repeats and a cysteine-rich DSL (Delta-Serrate-Lag-2) motif. Although in general these structural motifs are believed to participate in ligand-receptor interaction, it is not very clear what specific regions are involved in receptor binding and how they interact with the receptor. The function of the very short intracellular domain of Notch ligands is not clear either. Notch ligands have also been identified in mammals. *Jagged-1*, a rat homologue of *Serrate*, was cloned from Schwann cell cDNA library by low stringency hybridization(12). It has all the typical structures of Notch ligands. Jagged-1 is co-expressed with Notch1 in developing spinal cord and other tissues. Jagged-1 activated Notch1 blocks in vitro myogenesis in a way very similar to the truncated receptor. Other mammalian Notch ligands, such as human Jagged-1 and Jagged-2, mouse Delta-like 1 (Dll1) and Dll3 have also been cloned and shown to be involved in different developmental processes. For example, human Jagged-1 mutations have been found to be responsible for Alagille syndrome, a genetic disorder characterized by abnormal

development of liver, heart, skeleton, eye, face and kidney. However, the role of these mammalian Notch receptors in tumorigenesis has never been determined. *C. elegans* Apx-1, although has different receptor and different mutant phenotype from Lag-2, can fully substitute Lag-2 when expressed under the control of *lag-2* regulatory sequences(13). Similarly, *Drosophila* Serrate can functionally replace Delta during neuroblast segregation in the *Drosophila* embryo(14). This implicates that different Notch receptors and ligands function through a similar mechanism.

Genetic and molecular studies have also identified intracellular components of Notch signaling pathway. *Drosophila* gene *Suppresser of Hairless (Su(H))* may play a central role in Notch signaling. Su(H) protein is sequestered in the cytoplasm when co-expressed with Notch protein in cultured *Drosophila* S2 cells and is translocated to the nucleus when Notch binds to its ligand Delta(15). In mammalian cells, truncated mouse Notch1 and Notch2 have been shown to be localized in the nucleus and interacts directly with RBP-Jκ (recombination signal sequence binding protein for Jκ genes), a transcriptional factor highly related to Su(H). The binding of truncated Notch1 and Notch2 to RBP-Jκ activates the expression of RBP-Jκ repressed genes, such as Hairy Enhancer of Split (HES-1) (16, 17, 18). The fact that truncated Notch proteins without the extracellular domains are always localized to the nucleus has led to the speculation that a cleaved fragment of wild-type Notch receptor may participates directly in the downstream nuclear events of Notch signaling. A few studies have provided very strong evidence to support this model(19, 20).

Besides Su(H) and RBP-Jκ, not much is known about the intracellular regulation of Notch signaling. In collaboration with Dr. Iva Greenwald's lab, we identified a *C. elegans* gene *sel-10* as an intracellular negative regulator of *lin-12* activity(21). Sequence analysis indicates that SEL-10 contains one F-box domain and seven WD40 repeats, typical

features of a member of the CDC4 family of proteins, which are known to promote ubiquitin-mediated protein degradation. Previous studies on CDC4 demonstrate that CDC4 is a component of the ubiquitin-ligase complex, and its main function is to determine target specificity by binding to phosphorylated target proteins. CDC4 binding triggers the formation of covalent bond between ubiquitin and the target protein. Once ubiquitinated, the target protein can be rapidly degraded by 26S proteosome. Since, co-immunoprecipitation assays showed that C. elegans SEL-10 complexes with LIN-12 and with murine Notch4, we proposed that SEL-10 promotes the degradation of LIN-12/Notch proteins by the ubiquitin-proteosome pathway.

III. Purpose

The original goal of my proposed work is to identify Notch4 ligands and determine their role in mammary gland development and tumorigenesis.

The additional goal of my research is to understand the molecular mechanism underlying the regulation of Notch/LIN-12 by SEL-10.

Body

I. Technical Objectives

The first goal of my research is to identify ligands for mouse Notch4 receptor, understand the molecular details of their interactions with Notch4, and determine their involvement in mammary gland development and tumorigenesis. I proposed specific aims 1 and 2 to achieve this goal in a period of three years.

The second goal of my research is to study the functional and physical interactions between SEL-10 and Notch/LIN-12 receptors at the molecular level, and to provide evidence for our theory that SEL-10 can promote Notch/LIN-12 ubiquitination. I proposed specific aims 3, 4 and 5 for this goal.

Specific Aim 1: Identifying and Cloning Genes that Encode Putative Notch4 Ligands (MONTHS 1-18)

In mouse, several genes have been identified as Notch ligands, such as *Jagged* and *Delta-like* genes. Since some Notch ligands are interchangeable, we will test whether the protein products of these genes are able to interact with and activate Notch4 receptor.

In addition, we will try to identify novel putative ligands for Notch4 by a screening strategy. Extracellular domain of Notch4 receptor will be used as a molecular probe to screen an eukaryotic expression library from mouse mammary gland. Once a novel gene is identified, we will obtain its full length cDNA for further investigation.

Specific Aim 2: Biochemical and Biological Interactions between Putative Ligands and Notch4 Receptor (MONTHS 18-36)

To demonstrate that a candidate protein is the bona fide Notch4 ligand, we will first show its physical interaction with Notch4 receptor by molecular and cellular techniques. As a property of EGF repeat contain proteins, their interaction should be Ca^{++} dependent. We then plan to analyze the expression patterns of the candidate ligands and Notch4 receptor. This experiment will not only help us to rule out ligands for other Notch receptors that can cross-interact with Notch4 but also inform us whether Notch4 has different ligands in different tissues. Furthermore, by functional assays, we will test the candidate ligands for their ability to activate Notch4 signaling pathway. Finally, we will also test Notch4 ligands for their ability to regulate mammary epithelial cell development by using transformation and differentiation assays.

Specific Aim 3: Mapping the Domains Required for SEL-10/Notch Interaction.

I plan to use a series of deletion mutants of mouse Notch4 and human SEL-10 in coimmunoprecipitation assays to address which domains are involved in the binding between the two proteins. I will also test if Notch4 can be phosphorylated and if phosphorylation is required for Notch binding to SEL-1. I will learn from the results of these experiments whether the molecular nature of Notch/SEL-10 interaction is consistent with what has been established by the studies of CDC4.

Specific Aim 4: Ubiquitination of Notch Proteins.

To answer whether SEL-10 can promote Notch ubiquitination, I will first address if Notch can be ubiquitinated in the cell. One approach I will use is to test if the stability of

Notch proteins can be affected by specific inhibitors of proteosome. Another approach is to precipitate 6XHis tagged Notch proteins from the cell under denaturing conditions and probe for covalently linked ubiquitin with a ubiquitin specific antibody.

Specific Aim 5: Regulations of Notch Ubiquitination and Function by SEL-10.

Once I establish that Notch can be ubiquitinated in the cell, I will conduct in vitro ubiquitination assays to study if Notch ubiquitination can be mediated by SEL-10. I will also carry out a luciferase reporter assay to see if Notch activity can be regulated by SEL-10. To test if the WD40 repeats of SEL-10 can stabilize int-3 by blocking full length SEL-10 function, I will carry out pulse-chase experiment to study if the half-life of int-3 can be prolonged and if the steady state levels of int-3 can be increased by the WD40 repeats.

II. Experimental Results

This annual report describes the progress we have made during the first 24 months of this fellowship. The progress reported here will be related to the original tasks set out in the Statement of Work, and the newly added specific aims 3, 4 and 5.

Specific Aim 1: Identifying and Cloning Genes that Encode Putative Notch4 Ligands (MONTHS 1-18)

A. Obtaining full length cDNA clones of mouse Jagged and Delta-like 1. We have obtained rat Jagged-1 cDNA, epitope-tagged it and cloned the tagged gene into retroviral vector and adenoviral vector. At the same time, we also cloned Notch4 and int-3 genes into retroviral and adenoviral vectors. These vectors have been successfully used to drive stable

or transient gene expression in different cells. A Western blot showing the expression levels of the three proteins is attached in Appendix A.

We have also acquired mouse Developmental Endothelial Locus-1 (Del1) gene which encodes a EGF repeat containing protein in endothelial cells, the same location where Notch4 is expressed. We will test Del1 as a candidate Notch4 receptor. We are also in the process of acquiring more candidate genes, such as mouse Delta-like 1 (Dll1) and Dll3, Jagged-2, and so on. All these candidate Notch4 ligands will be tested for their ability to interact with Notch4 bio-chemically and biologically.

B. Screening mammary gland eukaryotic expression libraries for putative Notch4 ligand. For this approach, we planned to determine the locations where Notch4 ligands are most concentrated and then screen an expression library made from such locations. Northern blot and in situ hybridizations have shown that Notch4 is expressed in endothelial cells. To confirm this result, we have been trying to study the expression pattern of Notch4 in adult mouse tissues by immunohistochemistry. A well established expression pattern of Notch4 will be a good indication of where its ligands are located.

We used a rabbit polyclonal antiserum against the C-terminal region of Notch4 to probe for Notch4 expression in adult mouse kidney tissue sections. The characteristic glomeruli with endothelial cell clusters in the renal cortex are very easy to identify, and that makes kidney a perfect organ to establish the conditions of immunohistochemical analysis using our antiserum. Together with anti-Notch4 antibody, we also used pre-immune serum in our immunohistochemical staining as negative control and anti-PECAM (an endothelial marker) antibody as positive control. Our preliminary results strongly indicate that Notch4 is expressed in kidney glomeruli. However, high background has prevented us from producing publication quality photos. To solve this problem, we are now trying to

purify our anti-Notch4 antiserum using affinity chromatography and making antibodies against other regions of Notch4.

The Notch1 ligand, Jagged-1 has been reported to be expressed in endothelial cells. It is natural to address whether Jagged-1 can also serve as a Notch4 ligand. In our immunohistochemistry studies using kidney sections, we also include an affinity purified Jagged-1 antibody. The staining pattern of Jagged-1 is clearly endothelial and is very similar to Notch4 expression pattern.

Specific Aim 2: Biochemical and Biological Interactions between Putative Ligands and Notch4 Receptor (MONTHS 18-36)

A. Co-immunoprecipitation of Notch4 and its putative ligands. We are in the process of testing Notch4 and Jagged-1 in co-immunoprecipitation experiments. Jagged-1 has been HA-tagged and will be co-expressed with Notch4 by transient transfection into Bosc23 cells. Anti-Notch4 and anti-HA antibodies will be used to precipitate and detect the protein products.

B. Cell aggregation assay to show physical interactions between Notch4 receptor and ligands. This experiment has not been carried out yet. But we have gathered all the necessary reagents, and once a promising candidate gene is available, we will test it in this assay.

C. Transformation assays to show activation of Notch4 receptor by its ligands.

D. Differentiation assays to show activation of Notch4 receptor by its ligands.

We have not carried out experiments proposed in C and D. Instead, we have developed a luciferase assay to test Notch4 candidate ligands in a faster and more quantitative way. Once a Notch4 ligand is identified in the luciferase assay, we eventually will test its role in mammary gland development and tumorigenesis by transformation and differentiation assays using mammary epithelial cells. In our preliminary studies, we were unable to show conclusively whether Jagged-1 can activate Notch4 and lead to luciferase expression. We are in the process of modifying the experiment to get more consistent results.

E. Immunofluorescent staining to study the subcellular localization of Notch4 receptor before and after its activation by ligand. Recently studies have provide quite convincing evidence supporting the model that Notch activation by its ligands induces a proteolytic processing resulting in the translocation of the intracellular domain of the receptor to the nucleus. Only a small amount of the truncated protein is required to exert its nuclear function. That is why it is very hard to detect the nuclear fragment caused by ligand binding. We still plan to do the proposed immunofluorescent staining experiment at a convenient time. But it will not be a top priority in the overall plan.

F. Luciferase assays to show activation of Notch4 receptor by its ligands. This experiment was not proposed in my original proposal. The intracellular domain of Notch1 has been shown to be able to activate a luciferase reporter gene under the control of a HES-1 promoter(22). We replaced Notch1 intracellular domain with int-3 or Notch4 and showed that int-3 can activate HES-1 transcription while Notch4 can not. We are going to co-express Jagged-1 and Notch4 in HELA cells using adenovirus and determine if Jagged-1 can activate Notch4 and lead to luciferase expression. This experiment is much faster and easier to carry out than transformation and differentiation assays. It will be our major approach to identify Notch4 ligands.

G. Cross-regulation among Notch receptors and their ligands. This experiment was not proposed in my original proposal. In our experiments designed to study the function of Notch receptors in endothelial cells, we found that the expression of one Notch receptor or ligand can sometimes up-regulate or down-regulate the expression levels of other Notch receptors or ligands. For example, we have found by Northern blot that Notch1, Notch3, Notch4 and Jagged-1 are all expressed in RBE4 cells, a rat brain endothelial cell line. Exogenous expression of the activated form of Notch4, int-3 increases the steady state levels of both endogenous Notch4 and endogenous Jagged-1 (Appendix B). We will assess this cross-regulation in further details and try to determine the specificity among Notch ligands and receptors. This study has the potential to develop into a new functional analysis system for Notch ligand-receptor interaction.

Specific Aim 3: Mapping the Domains Required for SEL-10/Notch Interaction

A. Interaction between int-3 and human SEL-10. I have shown through coimmunoprecipitation assays that int-3 can not only bind to C. elegans SEL-10 (Appendix C), it can also form a complex with human SEL-10(Appendices D, E, F). The experiment was done by transfection of 293T cells with HA-tagged int-3 and myc-tagged human SEL-10, followed by immunoprecipiation and Western blotting using the corresponding antibodies.

B. Domains of int-3 and human SEL-10 required for their interaction. I generated a series of deletion mutants of int-3 and human SEL-10 and tested them in co-IP assays to investigate which domains are responsible for the binding between int-3 and human SEL-10. My results indicate the C-terminal region of int-3 after the CDC10/ankyrin repeats and

the WD40 repeat region of human SEL-10 are the domains involved in their interaction (Appendices D, E, F).

C. Interaction between human SEL-10 and SKP1. Since we proposed that SEL-10 targets int-3 for ubiquitination, we tested if the F-box domain of human SEL-10 could bind to SKP1, a component in the ubiquitination machinery, as suggested by studies on CDC4. Through co-IP assays, I demonstrated that human SEL-10 indeed binds to SKP1 through the F-box domain (Appendix G). This result further strengthens our model of how SEL-10 regulates Notch.

D. Phosphorylation of int-3 proteins. My preliminary data have demonstrated that int-3 is a phosphorylated protein and SEL-10 binds to phosphorylated int-3 better that the unphosphorylated form (data not shown). This is consistent with the model that CDC4 family proteins only recognize phosphorylated target proteins and therefore, ubiquitination is usually triggered by the phosphorylation of the target protein.

Specific Aim 4: Ubiquitination of Notch Proteins.

A. Steady-state levels of int-3 proteins under proteosome inhibitor treatment. My results demonstrated that treatment by lactacystin, a specific inhibitor of proteosome, could increase the steady-state levels of the C-terminal fragment of int-3 (int-3C) downstream of the CDC10/ankyrin repeats (Appendix H), strongly suggesting that Notch proteins can be ubiquitinated in the cell.

B. Covalent association between ubiquitin and int-3 proteins. This experiment is in progress. I plan to precipitate 6Xhis tagged int-3 proteins under denaturing conditions using Ni^{2+} charged beads, then probe the precipitates with anti-ubiquitin antibody. Since

the association between ubiquitin and its target protein is through covalent bond, denaturing conditions should preserve the complex between int-3 and ubiquitin while getting rid of other int-3 associated proteins, therefore, preventing false positive results and high level of background signals.

Specific Aim 5: Regulations of Notch Ubiquitination and Function by SEL-10.

A. in vitro ubiquitination. We will carry out in vitro ubiquitination assays by using int-3 and SEL-10 immunoprecipitates from Sf9 cells and other purified components of the ubiquitination pathway.

B. Regulation of the steady-state levels of int-3 by SEL-10. My preliminary data have shown that the WD40 repeat region of SEL-10 can increase the steady-state levels of int-3C protein in a co-transfection experiment (Appendix H). The result is similar to that seen when int-3C expressing cells were treated with lactacystin, suggesting that the WD40 repeats have dominant negative effects on endogenous SEL-10.

C. Regulation of int-3 stability by SEL-10. I plan to address whether the half-life of int-3 proteins can be regulated by SEL-10 or its deletion mutants by using pulse-chase labeling experiment.

D. Regulation of int-3 activity by SEL-10. I have shown in previous experiments that int-3 can activate a luciferase reporter gene under the control of HES-1 promoter. I will include SEL-10 or its deletion mutants in this assay to study if SEL-10 can have a direct effect on int-3 mediated biological events.

Conclusions

Data presented in the first half of this annual report represent our progress in the experiments outlined in the specific aims of the original research proposal. In the past year, we developed a series of reagents that will enable us to carry out biochemical and biological studies. We have also developed functional analysis systems in which we can quickly and easily test a candidate protein for its ability to bind and activate Notch4 receptor. Our observation that cross-regulations exist among different Notch pathway genes brings up an interesting angle to understand Notch ligand-receptor interaction and the potential to develop a new assay for ligand-dependent Notch activation.

In addition to the studies of Notch receptor, I also investigated the regulation of Notch signaling by *sel-10* gene. My results have demonstrated that the intracellular domain of Notch can bind to the WD40 repeats of SEL-10, in a seemingly phosphorylation-dependent manner. The F-box region of SEL-10 binds to other components of the ubiquitination, indicating that SEL-10 negatively regulate Notch function by targeting it for ubiquitin-mediated degradation. I also provided preliminary results showing that Notch is a ubiquitinated protein in the cell, and its ubiquitination and stability can be regulated by SEL-10.

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Appendices

Appendix A Western blots of int-3, Notch4 and Jagged-1 proteins

Appendix B Northern blot analysis on RBE4 cells

Appendix C Interactions between int-3 and C. elegans SEL-10

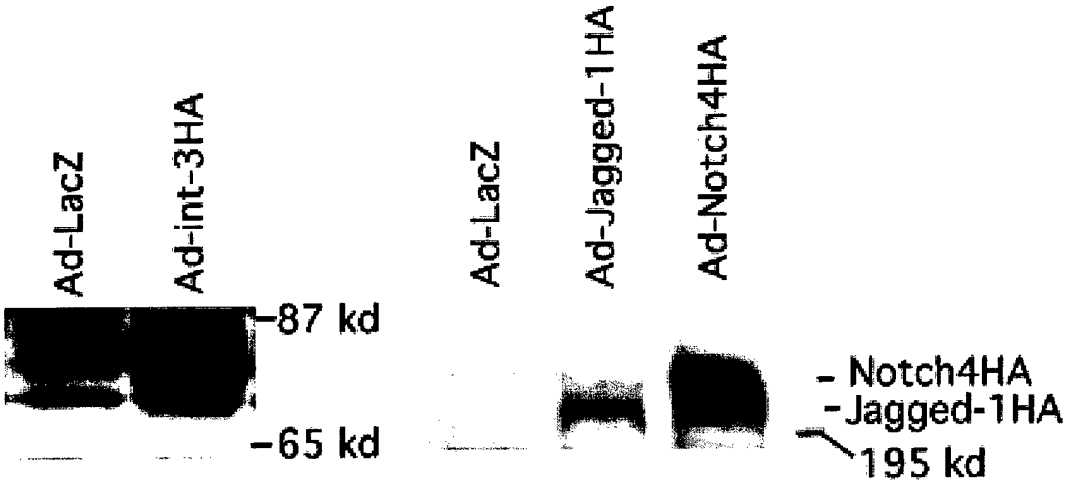
Appendix D Domains of int-3 and human SEL-10 involved in their interaction

Appendix E Interaction between human SEL-10 and SKP1

Appendix F Stabilization of int-3 by lactacystin, a proteosome inhibitor

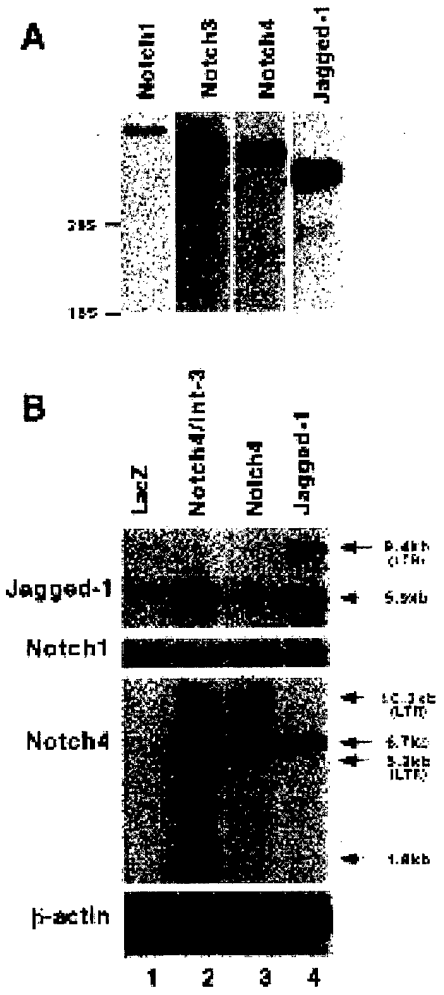
Appendix G Stabilization of int-3 by the WD40 repeats of human SEL-10

Appendix A



A. HELA cells infected by adenoviruses (Multiplicity of Infection=10) carrying *LacZ, int-3HA, Notch4HA* and *Jagged-1HA* genes.

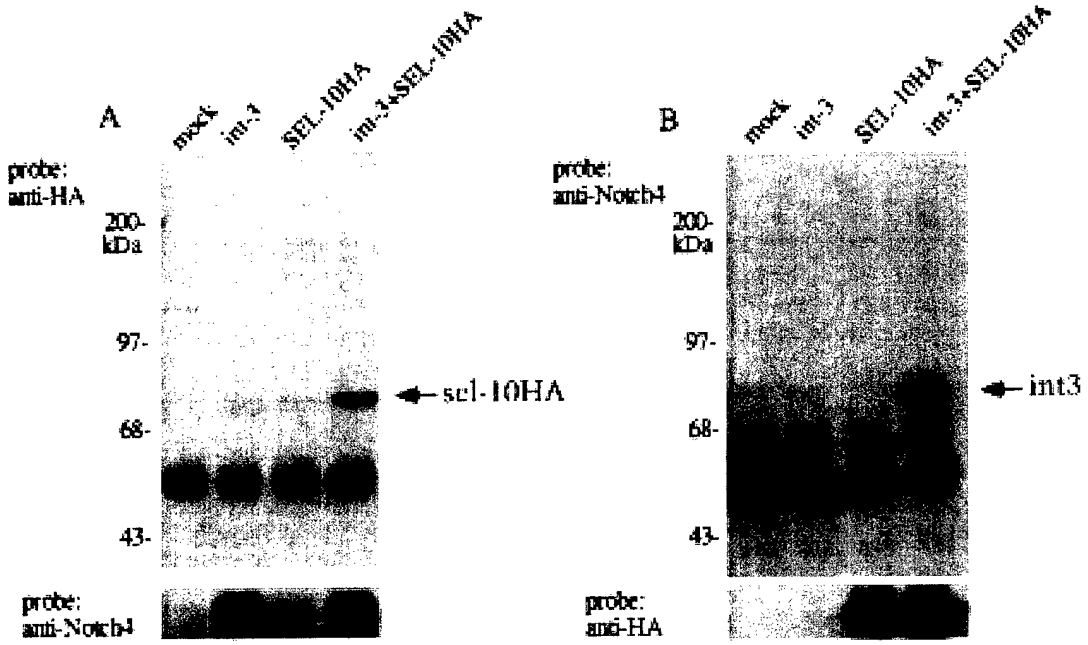
Appendix B



A. Notch1, Notch3, Notch4 and Jagged are all expressed in RBE4 cells. 40ug of total RNA from RBE4 cells hybridized to riboprobes for either *Notch1, Notch3, Notch4* or *Jagged-1*

B. Cross-regulation of Notch receptors and Ligand. 40ug of total RNA (lane 1, 3) or 20ug of total RNA (lane 2, 4) from RBE4 cells expressing LacZ, int-3, Notch4 or Jagged-1 from a CMV promoter, was hybridized to riboprobes for *Jagged-1, Notch1, Notch4* or *β-actin*. int-3 expression up-regulates the expression of endogenous Notch4 and Jagged-1. RBE4 cells stably expressing Notch receptor or ligand were generated by retroviral infection. RNA labeled with "LTR" indicates retroviral genome RNA that is transcribed from the LTR promoter.

Appendix C



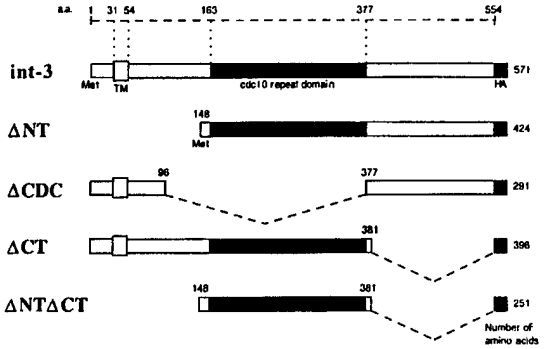
Co-immunoprecipitation of int-3 and C. elegans SEL-10.

A. Cell extracts expressing int-3 or C.elegans SEL-10HA proteins were immunoprecipitated with anti-Notch4 antibody, then probed with anti-HA to visualized the SEL-10HA brought down by int-3, or with anti-Notch4 to confirm that int-3 was properly precipitated.

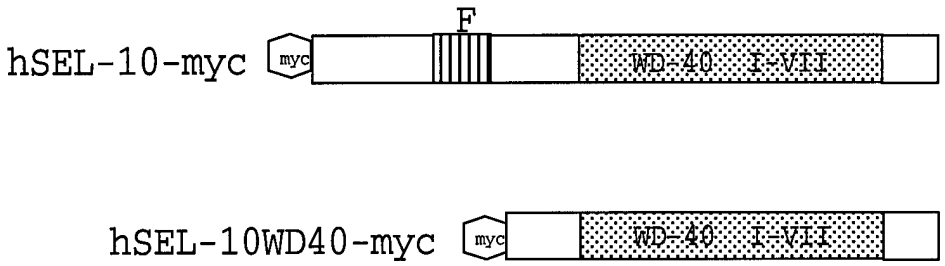
B. The same cell extracts as in A were immunoprecipitated with anti-HA antibody and then probed with either anti-Notch4 or anti-HA antibody, to confirm that int-3 can also be brought down by SEL-10HA.

Appendix D

A



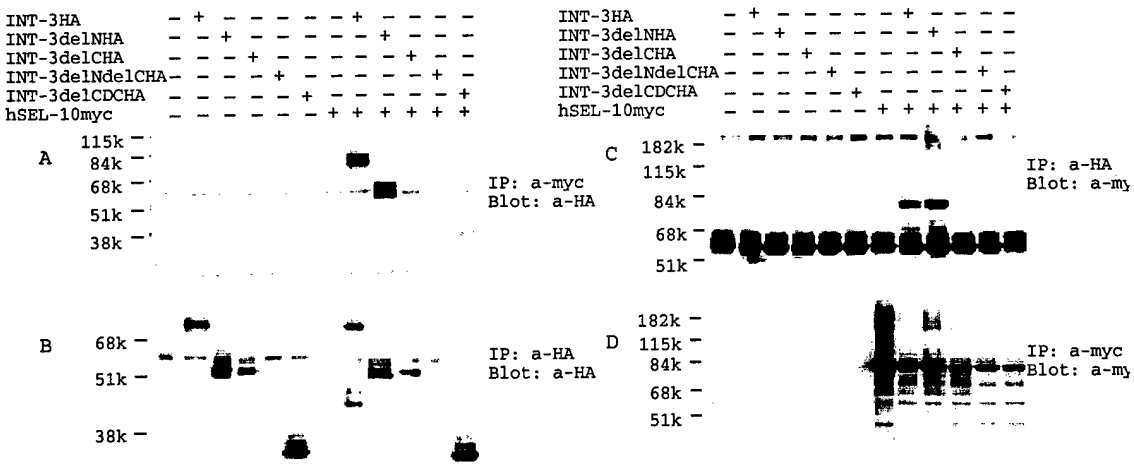
B



Schematic Map of int-3 and hSEL-10 deletion mutants used in co-immunoprecipitation assays.

A. int-3 deletion mutants. All proteins are HA-tagged at the C-terminus.
B. human SEL-10 deletion mutants. Both proteins are myc-tagged at the N-terminus.

Appendix E

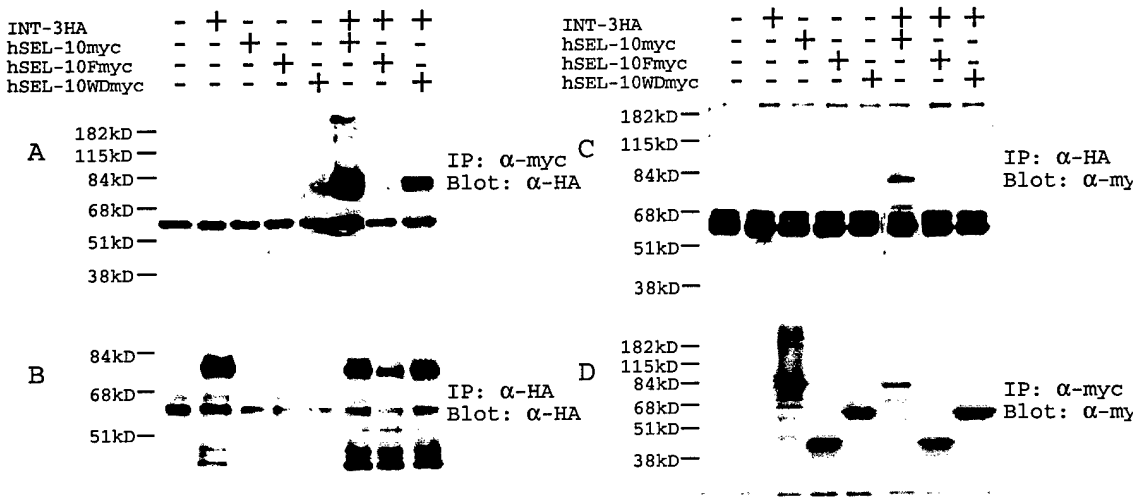


Interactions between full-length human SEL-10 and int-3 deletion mutants.

A, D. 293T cells transfected with myc-tagged human SEL-10 and one of a series of HA-tagged int-3 proteins were immunoprecipitated with anti-myc antibody, then probed with either anti-HA antibody to visualize the int-3 proteins brought down by hSEL-10, or with anti-myc antibody to confirm the proper immunoprecipitation of hSEL-10myc.

C, B. The same samples as in A and D were immunoprecipitated with anti-HA antibody and then probed anti-myc or anti-HA antibodies to confirm the interaction between hSEL-10 and int-3 proteins.

Appendix F

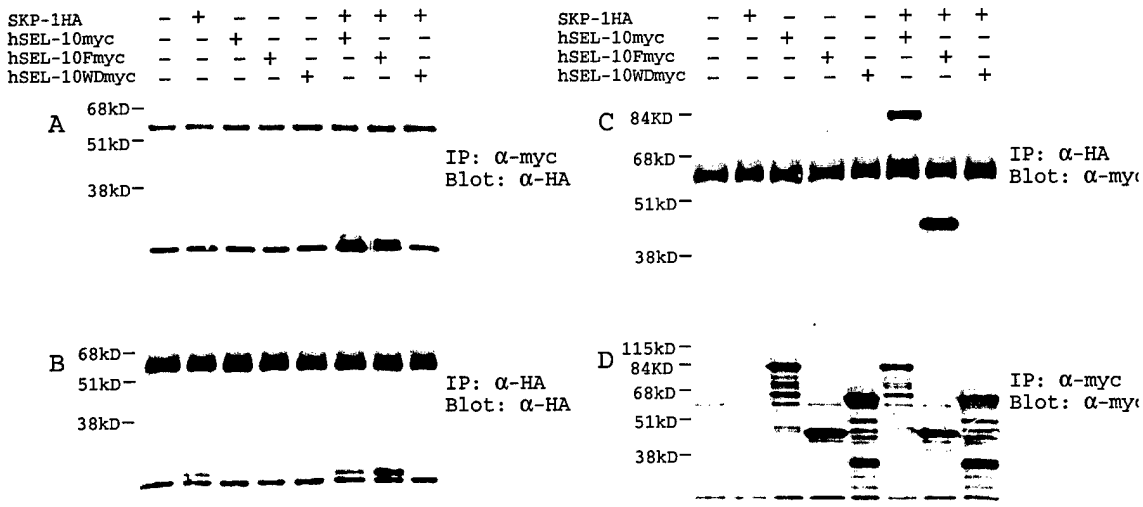


Interactions between int-3 and SEL-10 deletion mutants

A, D. 293T cells expressing int-3HA and myc-tagged SEL-10 proteins were immunoprecipitated with anti-myc antibody, then probed with either anti-HA to visualize int-3HA brought down by SEL-10 proteins, or with anti-myc to confirm the proper precipitation of SEL-10 proteins.

C, B. The same extracts as in A and D were immunoprecipitated with anti-HA antibody, then probed with either anti-myc or anti-HA antibodies to confirm the interaction between int-3HA and SEL-10 proteins.

Appendix G

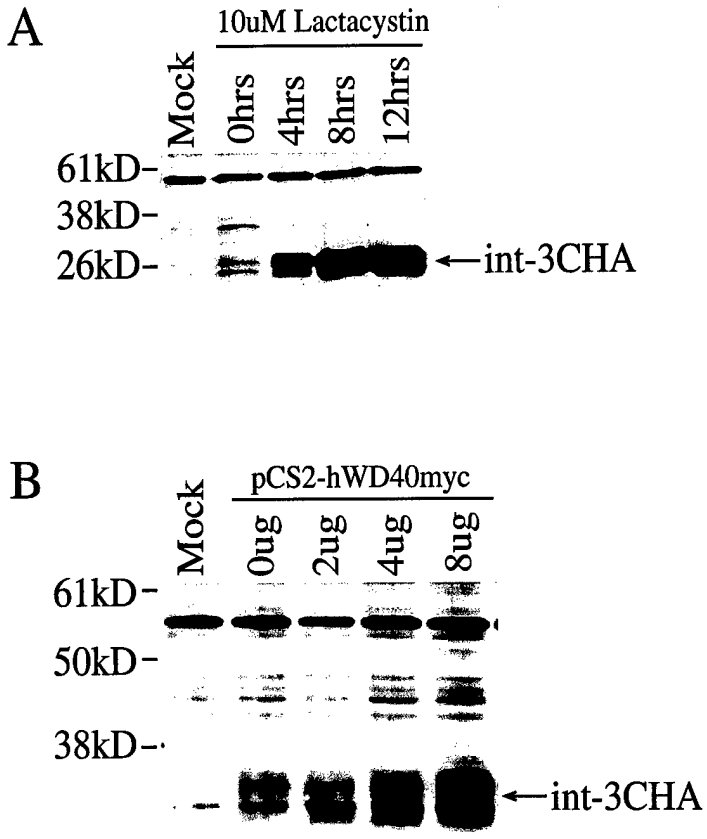


Coimmunoprecipitation of SEL-10 and SKP-1

A, D. 293T cells expressing SKP-1HA or/and myc tagged SEL-10 proteins were immunoprecipitated with anti-myc antibody and then probed with either anti-HA or anti-myc antibody.

B, C. The same samples as in A and D were immunoprecipitated with anti-HA antibody and then probed with either anti-myc or anti-HA antibodies.

Appendix H



Stabilization of int-3CHA protein by lactacystin or WD40 repeats.

A. 1ug of pQNCint-3CHA plasmid was transfected into each well on a 6-well plate by using calcium phosphate method. Starting 24 hours post-transfection, lactacystin was added to medium at different time points at a final concentration of 10uM. Cells in different wells were harvested together and lysed for Western blot analysis.

B. 2ug of pQNCint-3CHA plasmid was transfected into each 60-mm plate, except the mock plate. Various amounts of pCS2hWD40myc plasmid encoding the WD40 repeats of human SEL-10 was co-transfected with the int-3CHA plasmid. Cells were harvested two days after transfection for Western blot analysis.

Each lane of the Western blots contains 20ug of total cell extract.